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Critical Epitopes in Transmissible Gastroenteritis Virus Neutralization

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Purified transmissible gastroenteritis (TGE) virus was found to be composed of three major structural proteins having relative molecular weights of 200,000, 48,000, and 28,000. The peplomer glycoprotein was purified by affinity chromatography with the monoclonal antibody (MAb) 1D.G3. A collection of 48 MAbs against TGE virus was developed from which 26, 10, and 3 were specific for proteins E2, N, and E1, respectively. A total of 14 neutralizing MAbs of known reactivity were E2 protein specific. In addition, MAb 1B.C11, of unknown specificity, was also neutralizing. These MAbs reduced the virus titer 10^2- to 10^9-fold. Six different epitopes critical in TGE virus neutralization were found, all of which were conformational based on their immunogenicity and antigenicity. Only the epitope defined by MAb 1G.A7 was resistant to sodium dodecyl sulfate treatment, although it was destroyed by incubation in the presence of both the detergent and β-mercaptoethanol. The frequency of MAb-resistant (*mar*) mutants selected with four MAbs (1G.A7, 1B.C11, 1G.A6, and 1E.F9) ranged from 10^{-6} to 10^{-7}, whereas the frequency of the putative *mar* mutant defined by MAb 1B.B11 was lower than 10^{-9}. Furthermore, the epitopes defined by these MAbs and by MAbs 1H.C2 and 1A.F10, were present in 11 viral isolated with different geographical locations, years of isolation, and passage numbers (with the exception of two epitopes absent or modified in the TOY 56 viral isolate), suggesting that the critical epitopes in TGE virus neutralization were highly conserved.

Transmissible gastroenteritis (TGE) virus of swine is a member of the *Coronaviridae* family that causes a disease of the gastrointestinal tract, producing transitory enteritis in adult animals and 80 to 100% mortality in animals less than 10 days old (6; for reviews, see references 51 and 53). The virus infects epithelial cells and, in some cases, lung macrophages (33).

The viral particle is spherical with a diameter of around 100 nm, and its lipidic envelope covers a nucleocapsid with helical symmetry, formed by an 18.5-kilobase, positive, single-stranded RNA that is infectious after single-hit kinetics (8, 28). The viral particle is composed of two glycoproteins (E2 and E1) and one nucleoprotein (N). The E2 glycoprotein is hydrophobic (28), induces neutralizing antibodies (18), and forms club-shaped surface projections. E1 is a transmembrane protein (18–20).

TGE virus is serologically related to canine coronavirus, feline infectious peritonitis virus, and human coronavirus 229E, being neutralized by antisera to the first two (27). TGE virus-specific polyvalent antibodies do not react with porcine epidemic diarrhea virus, but the precise serological relationship between these two coronaviruses is unknown (45; G. Jiménez and L. Enjuanes, unpublished results).

Thus, the development of a collection of monoclonal antibodies (MAbs) that are TGE virus specific would facilitate the study of the serological relationship between TGE virus and other coronaviruses, its neutralization mechanism, antigenic variability, and the role of viral protein structure and humoral immune response in host protection.

In the present study a library of MAbs specific for the structural proteins of TGE virus was generated. These MAbs were used to study (i) the number of critical epitopes in TGE virus neutralization, (ii) their immunogenicity and antigenicity, and (iii) the variability of these epitopes, by isolating *mar* mutants and studying the presence of the critical epitopes in viral isolates with different origins and dates of isolation.

MATERIALS AND METHODS

Cells. The epithelial swine testicle (ST) cell line developed by McClurkin (38) was obtained from Dr. Kemeny, National Animal Disease Center, Ames, Iowa. Cells were grown as monolayers in growth medium consisting of Dulbecco modified Eagle (DME) medium (GIBCO Europe) and 10% newborn calf serum (Flow Laboratories, Inc.).

Animals. BALB/c mice, originally obtained from R. A. Fox, Frederick Cancer Research Center, Frederick, Md., were used for immunization and as a source of thymocytes and peritoneal macrophages, when the mice were approximately 10, 5, and 16 weeks old, respectively.

Viruses. The characteristics of all the TGE virus isolates used in this study are summarized in Table 1.

Virus titration and plaque isolation. TGE virus was titrated on ST cells as described by Brian et al. (8), with minor modifications. Briefly, cells were seeded in 24-well tissue culture plates (Costar). Serial dilutions (10-fold) of virus were made in DME medium containing 2% newborn calf serum and 40 μg of DEAE-dextran per ml (Pharmacia). Portions containing 50 μl of each dilution were applied to cells. After 1 h of virus adsorption, the inoculum was replaced with medium containing 2% newborn calf serum, 40 μg of DEAE-dextran per ml, and 0.1% agarose, and the cells were incubated at 37°C for 2 to 3 days in a humidified CO_2 incubator. Cells were fixed with 10% formaldehyde and stained with 0.1% crystal violet, and the plaques were counted.

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TABLE 1. TGE virus isolates

Designation	Origin (year of isolation)	Characteristics[a]	Source (reference)
PUR 54-C1.P1	Purdue University (1954 or earlier)	ATCC VR-763 antigen for MAb production cloned and passaged on PK cells; attenuated	E. H. Bohl (5, 7, 35)
PUR 54-C1.P30	Purdue University (1954 or earlier)	Derived from PUR 54-C1.P1 by 30 passages on ST cells	This publication
MIL 65-C1.P1	USA (1965 or earlier)	ATCC VR-743 passaged 13 times in PK and 5 times in gnotobiotic pigs; virulent	American Type Culture Collection (5, 7)
NEB 82-UC	Nebraska (1982 or earlier)	Attenuated live vaccine	A. Torres-Medina (personal communication)
VAC MVP	USA (unknown)	Attenuated live vaccine	Modern Veterinary Products, Inc.
HOR 49-C1.P1	Minnesota (1949)	Received after being passaged 14 times in young pigs	R. Wesley (61)
SHI 56-19.C1.P1	Japan (1956)	Received at passage 19 on swine kidney cells; virulent	H. Sazawa (16, 23)
SHI 56-25.C1.P1	Japan (1956)	Received at passage 25 on swine kidney cells	R. Wesley (23)
SHI 56-83.C1.P1	Japan (1956)	Received at passage 83 on swine kidney cells; attenuated	H. Sazawa (16, 24)
TOY 56-163.C1.P1	Japan (1956)	Received at passage 163 on swine kidney cells; attenuated	H. Sazawa (16)
BRE 79 (D52)	France (1979)	Virulent	P. Vannier (1)

[a] All viruses were cloned three times by plaque isolation on ST cells before use, except PUR 54-C1.P30, NEB 82-UC, VAC MVP, and BRE 79 (D52).

For plaque isolation a method similar to the one described by Takayama and Kim (54) was followed with minor modifications. ST cells in petri dishes (diameter, 35 mm) were inoculated with 0.2 ml of serial 10-fold dilutions in the medium described above. After 1 h of virus adsorption, the inoculum was replaced with DME medium containing 2% newborn calf serum and 0.6% agar (Agar Noble, Difco Laboratories). Isolated plaques were selected 3 days later and diluted in 0.5 ml of fetal calf serum, and the monolayer was stained with crystal violet to ensure the absence of other plaques in the proximity of the selected one. The procedure was repeated three to five times before stocks of each virus clone were grown.

Virus purification. The procedure for virus purification will be described elsewhere (I. Correa, M. P. Melgosa, G. Jiménez, M. J. Bullido, and L. Enjuanes, submitted for publication). Briefly, ST cells were grown in roller bottles and incubated with virus at a multiplicity of infection of 10 PFU per cell. The virus from clarified culture supernatants was concentrated by centrifugation through a discontinuous sucrose gradient consisting of two layers of 30 and 45% sucrose in 0.01 M Tris hydrochloride–1 mM disodium EDTA–1 M NaCl (pH 7.4) (TEN buffer) supplemented with 0.2% Tween 20 for 1.5 h at 25,000 rpm and 4°C in a Sorvall AH-627 rotor.

The fraction collected at the interface was diluted with 0.3 volumes of TEN and sedimented through a 30 to 42% sucrose gradient containing TEN at 25,000 rpm for 2 h at 4°C in the same rotor. The gradient was fractionated and a 1.185- to 1.195-g/cm^3 density zone containing the virus was selected, diluted in 0.5 volumes of TEN, and concentrated by sedimentation as described above.

RIA. For radioimmunoassay (RIA), TGE virus protein (0.25 μg per well in 50 μl of phosphate-buffered saline) was adsorbed to polyvinyl disposable "U" plates (Dynatech Laboratories, Inc.), by overnight incubation at 37°C. The subsequent steps of the assay were performed as described by Nowinski et al. (43).

Hybridoma production and selection. To obtain hybridomas secreting TGE virus-specific MAb, cells from the mouse myeloma cell line X63/Ag 8.653 (30; provided by L. Cicurel, Wistar Institute, Philadelphia, Pa.) were fused with spleen cells from BALB/c mice immunized with purified TGE virus by a protocol of intensive immunization described previously (52). The hydridizations were carried out as described by Nowinski et al. (43) and Sanz et al. (50). By this procedure, 80 to 100% of the wells were found to contain viable hybrids. The hybridomas and MAbs were named as described previously (50). The screening to detect hybridomas reacting to TGE virus was performed by RIA. All hybridomas were cloned at least three times.

Determination of MAb isotypes. The class and subclass of the MAbs were determined by immunodiffusion (44) with antisera specific for mouse heavy and light chains, purchased from Research Products, Inc., or Litton Bionetics.

Neutralization assay. A plaque reduction assay was performed by incubating 10^4 to 10^9 PFU of TGE virus in 50 μl of phosphate-buffered saline with 2% fetal calf serum in the presence of 50 μl of hybridoma culture supernatants at room temperature for 30 min. When indicated, 50 μl of rabbit serum was added as a source of complement and the incubation was continued for a further 30 min. Dilutions (10-fold) of the virus-MAb mixture in DME medium containing 2% fetal calf serum were carried out. A 50-μl sample was plated from each dilution, and the number of PFU was determined as described above. Virus samples were incubated with control, medium, or nonneutralizing MAbs (NMAbs) and processed by the same protocol. The neutral-

ization index of each hybridoma supernatant was determined by dividing the number of PFU of virus per ml mixed with normal medium by the number of PFU of virus per ml in the presence of a given MAb, and this index is expressed as the $\log_{10}$ of this ratio.

Immunoadsorption assay. The specificity of the MAbs was determined by immunoadsorption of the corresponding viral antigen on MAb-coated plates, by a procedure described by Melero and González-Rodríguez (39). Labeled antigens were prepared from purified TGE virus grown on ST cell monolayers in the presence of 20 μCi of [^{35}S]methionine per ml (1,300 Ci/mol; Amersham Corp.) in DME medium with the methionine concentration reduced 10-fold. Purified, labeled virus (200 to 400 μg of virus in 0.2 ml of phosphate-buffered saline) was dissociated by incubation at 37°C for 1 h, with 5 volumes of virus dissociation buffer (VDB; 50 mM Tris hydrochloride [pH 7.5], 5 mM disodium EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate) in the presence of 0.1 mM L-1-tosylamide-2-phenylethylchloromethyl ketone–0.1 mM *N*-α-*p*-tosyl-L-lysinechloromethyl ketone–1 mM phenylmethylsulfonyl fluoride–1 μg of pepstatin per ml. Solubilized antigens were diluted in 20 volumes of VDB diluted 1:20 in water and incubated for 30 min at 37°C, after which nondissociated material was removed by centrifugation at 140,000 × *g* for 2 h at 4°C. Generally this procedure solubilized 70 to 80% of [^{35}S]methionine-labeled TGE virus. Viral antigen samples with 20×10^3 to 40×10^3 cpm in 50 μl of medium were incubated in MAb-coated wells to perform the immunoadsorption assay (39). Briefly, polyvinyl disposable U plates (Dynatech) were coated by (i) incubating the plates with 5 μg of protein A (Pharmacia) per well, (ii) saturating the remaining binding sites with bovine serum albumin, and (iii) incubating the plates with rabbit antibodies specific for mouse immunoglobulins to capture MAbs with different isotypes. After ^{35}S-labeled viral antigens, prepared as described above, were added, the plates were incubated overnight after which they were washed with a buffer containing Nonidet P-40, and the bound antigens were detached with sodium dodecyl sulfate (SDS)-containing buffer before being resolved by SDS-polyacrylamide gel electrophoresis (PAGE). The gels were prepared for autoradiography by using sodium salicylate (10) and were dried and exposed on RP-X1 films (Mafe) at −70°C.

Immunoblotting analysis. The binding of polyclonal or monoclonal antibodies to viral proteins transferred to nitrocellulose paper was performed by the method of Towbin et al. (56) with minor modifications. Briefly, the proteins were resolved by SDS-PAGE by the method of Laemmli (31) in the presence or absence of β-mercaptoethanol (β-ME). The gel was equilibrated with 20% methanol in 25 mM Tris base–192 mM glycine (pH 8.3) after which the poteins were electrophoretically transferred to a nitrocellulose membrane filter at the field strength of 7.5 V/cm for 5 h at 4°C. After the transfer, the nitrocellulose membrane was washed in 500 mM NaCl–20 mM Tris hydrochloride (pH 7.5) (TBS) after which the incubation was continued with TBS containing 5% nonfat dry milk (Molico, Nestlé) as described by Johnson and Elder (29), to saturate remaining protein binding sites. The membrane was then incubated in hybridoma supernatant or a 1:40 dilution of the antiserum in TBS containing 1% nonfat dry milk for 12 h at 4°C. After the membrane was washed in TBS containing 0.05% Tween 20, it was incubated with peroxidase-labeled rabbit immunoglobulins specific for mouse immunoglobulins for 1 h at room temperature. After 1 h of washing in TBS with 0.05% Tween 20, bands specifically recognized by the antisera or the MAbs were developed by the 4-chloro-naphtol technique (25).

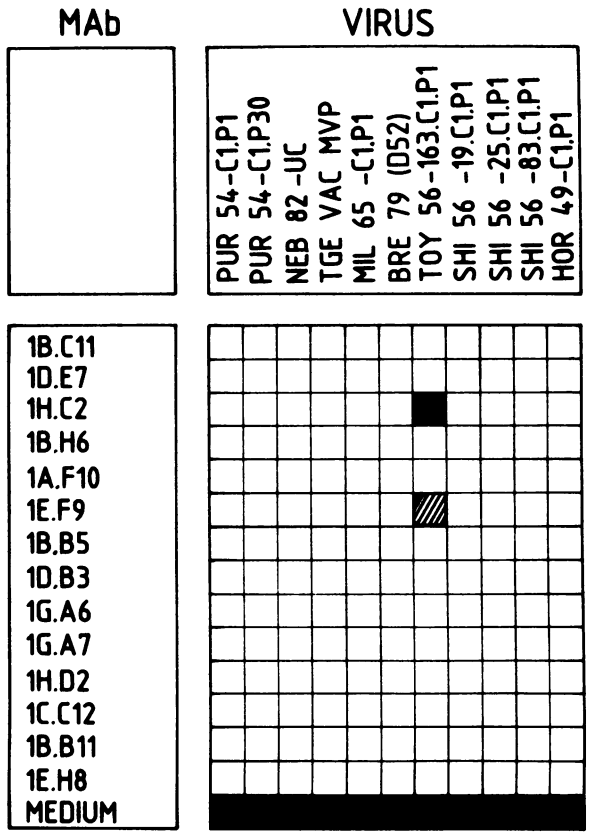

FIG. 1. Conservation of critical epitopes in TGE virus isolates. From each virus isolate, 10^2, 10^3, or 10^4 PFU were neutralized by the indicated MAbs, as described in Materials and Methods. The characteristics and origin of the viral isolates are shown in Table 1. Empty, hatched, and full squares represent a reduction of $>10^4$, between 10^4 and 10^1, or $<10^1$ PFU, respectively.

Purification of E2 glycoprotein. The purification of the peplomer-forming protein of TGE virus will be described in detail elsewhere (Correa et al., submitted for publication). Briefly, purified TGE virus was disrupted in VDB or a buffer with 1% SDS in the presence of protease inhibitors. Nondissociated material was eliminated by centrifugation, and diluted supernatants were chromatographed through an affinity column prepared with MAb 1D.G3 specific for E2. The purified protein was dialized overnight against 50 mM ammonium acetate and was lyophilized.

Typification of viral isolates. Typification of viral isolates was achieved by studying the neutralization of different viral isolates with a panel of TGE virus-specific NMAbs, which recognized at least six different epitopes. Viral isolates with different geographical origins, dates of isolation, and passage numbers (Fig. 1), that were previously cloned (with the exception of virus PUR 54-C1.P30 and the vaccinial strains NEB 82 and MVP) were grown on ST cells and harvested at 24 h postinfection. Supernatants were clarified, and 50-μl samples containing 10^2, 10^3, and 10^4 PFU were incubated with 1 volume of the indicated hybridoma supernatants, as described above for the neutralization assay, after which the extent of the neutralization was recorded. A hybridoma supernatant containing MAb 6A2, specific for Moloney

TABLE 2. Frequency of *mar* mutants for different epitopes of TGE virus envelope E2 glycoprotein

TGE *mar* mutant	Frequency
1B.B11	$<10^{-9.0}$
1B.C11	$10^{-7.0}$
1G.A6	$10^{-6.2}$
1E.F9	$10^{-6.0}$
1G.A7	$10^{-6.0}$

leukemia virus protein p30 (Cicurel et al. [11]) was used as a negative control.

Selection of *mar* mutants. The selection of *mar* mutants with five MAbs (Table 2) was attempted by incubating about 10^9 PFU of cloned TGE virus in 0.1 ml of medium with 2 volumes of each hybridoma supernatant in the absence of complement, as described above for the neutralization assay. Then ST cell monolayers growing in petri dishes (diameter, 35 mm; Costar) were inoculated with 0.2-ml portions of 10-fold dilutions of the virus-MAb mixtures and incubated for 1 h at 37°C, before an agar overlay like the one described above for the plaque assay, containing 0.5 ml of the corresponding hybridoma supernatant per dish, was added. At 2 to 3 days after infection, plaques were collected as described above, and the selection procedure was carried out two more times. Individual plaques were used to infect monolayers of ST cells growing on 25-cm^2 flasks (Costar).

RESULTS

Purification of TGE virus and its major E2 glycoprotein. When the polypeptides from purified TGE virus were analyzed by SDS-PAGE, three major structural proteins of molecular weights 200,000, 48,000, and 28,000 (proteins E2, N, and E1, respectively) (18–20, 28, 32) were detected (Fig. 2, lane a). The protein content of the three bands accounted for more than 95% of all proteins detected in the gel (results not shown). The virus was homogeneous, as determined by velocity sedimentation, behavior in isopycnic gradients, electron microscopy of negatively stained specimens, and absence of cell contaminants, when purified from virus grown in cells prelabeled with [^{35}S]methionine (Correa et al., submitted for publication).

The viral proteins from the dissociated virus and the purified E2 protein are also shown in Fig. 2 (lanes b and c, respectively). The E2 protein selected by affinity chromatography after dissociation of TGE virus with 1% SDS had a purity of higher than 96% as determined by gel densitometry (results not shown).

Production and general properties of TGE virus-specific MAbs. The specificity, isotype, titer, and virus neutralization index of the 48 MAbs specific for TGE virus proteins produced from five independent fusions are shown in Table 3. From the isolated MAbs, 26, 10 and 3 were specific for E2, N, and E1 glycoproteins, respectively, as determined by immunoadsorption assays (Fig. 3). The other nine MAbs did not appear to react with the viral proteins either in the immunoadsorption assay or the immunoblotting analysis.

The most frequent isotype was immunoglobulin G1 (IgG1) (32 MAbs), followed by IgG2a, IgG2b, and IgM (present in 8, 3, and 2 MAbs, respectively). The MAb titers, determined by RIAs of culture supernatants and ascites fluids, ranged from 10^1 to 10^5 and from 10^3 to 10^8, respectively.

Critical antigen in TGE virus neutralization. All 14 MAbs of known specificity that were able to neutralize TGE virus infectivity more than 10-fold in the absence of complement were E2 specific (Table 3), indicating that this was the critical antigen in TGE virus neutralization. In the presence of rabbit serum, used as a source of complement, none of the MAbs produced a significant increase in the extent of TGE virus neutralization when undiluted hybridoma culture supernatants were used as the antibody source. The different NMAbs decreased the viral infectivity to a variable extent ranging from 10^2- to higher than 10^9-fold.

Nature of the critical epitopes for the induction of NMAbs. To determine the nature (conformational or denaturation-resistant) and the immunogenicity of the critical epitopes in TGE virus neutralization, the induction of polyvalent antibodies by native TGE virus or by the virus and the E2 glycoprotein, after denaturation with detergents and β-ME, was studied. The results (Table 4) indicate that, while native TGE virus or the purified E2 protein solubilized with Nonidet P-40 and sodium deoxycholate induced high titer antibodies as determined by both RIA (10^2 to $10^{3.5}$) and neutralization (neutralization index, >3.5), TGE virus denatured with SDS alone or in the presence of β-ME (or the E2 glycoprotein incubated in the presence of SDS and β-ME) also induced high antibody titers (10^2 to $10^{4.5}$) by RIA, but very low or nonsignificant levels (neutralization index, <0.4) of neutralizing antibodies.

The antigenicity of the critical epitopes in TGE virus neutralization was determined by studying the binding of the NMAbs to TGE virus proteins by immunoblotting assays. Representative results are shown in Fig. 4 and summarized in Table 5 for E2 glycoprotein-specific MAbs. The epitope recognized by non-NMAb 1D.G3 was resistant to denaturation by SDS plus β-ME, and the epitope(s) recognized by

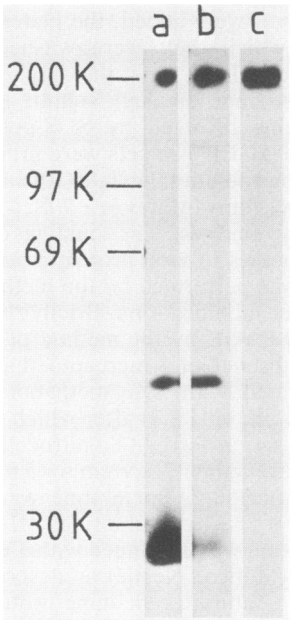

FIG. 2. PAGE of proteins from TGE virus dissociated with SDS and β-ME (a) or with Nonidet P-40 and sodium deoxycholate, unfractionated (b) or selected by affinity chromatography (c) with the E2 protein-specific MAb 1D.G3. [^{35}S]methionine-labeled virus was treated as indicated in Materials and Methods, the proteins were resolved by SDS-PAGE in an 8% polyacrylamide gel, and the gel was processed for autoradiography by the method of Chamberlain (10).

non-NMAb 6A.A6 and NMAbs 1G.A7 and 1A.F10 was resistant to denaturation by SDS in the absence of β-ME.

Variability of critical epitopes involved in TGE virus neutralization. An estimation of the antigenic variability of some of the critical epitopes involved in TGE virus neutralization was made by studying both the frequency of TGE virus *mar* mutants and the conservation of the critical epitopes in different TGE virus isolates. As shown in Table 2, the frequency of *mar* mutants for the TGE virus epitopes defined by five E2 protein-specific MAbs ranged from $10^{-6.0}$ to lower than $10^{-9.0}$. These values were obtained with TGE virus that was cloned and passaged twice on ST cells for virus stock production. It was not possible to isolate *mar* mutants by using MAb 1B.B11, as this antibody always neutralized all virus (10^9 PFU) used in the assay. All *mar* mutants isolated were able to produce 10^8 to 10^9 PFU/ml of culture medium, as could the wild type virus.

The frequencies of *mar* mutants shown (Table 2) for different epitopes of the E2 glycoprotein of TGE virus (see below) were 10^2- to 10^5-fold lower than the ones described for other RNA viruses (see Discussion). This suggested that

TABLE 3. MAbs specific for TGE virus proteins

Protein specificity:	MAb	Isotype	Titer[a]		Neutralization index[b]	
			Medium	Ascites	−Complement	+Complement
E2	1B.B11	IgG1 (K)	10^3	10^5	>4.5	>4.5
	1B.C11	IgG1 (K)	10^4	10^7	>4.5	>4.5
	1G.A6	IgG1 (λ_1)	10^4	10^7	>5.1	>5.3
	1B.H6	IgG2a (K)	10^2	10^8	>5.5	>5.5
	1G.A7	IgG1 (K)	$10^{3.5}$	10^5	>5.5	>5.1
	1H.C2	IgG1 (K)	10^5	10^6	>5.5	>5.1
	1H.D2	IgG2a (K)	10^5	10^7	>5.1	>5.3
	1D.B3	IgG2a (K)	10^4	10^6	>4.7	>4.7
	1D.E7	IgG1 (K)	10^4	10^7	>4.5	>4.5
	1C.C12	IgG2a (λ_1)	10^3	10^5	>4.5	>4.5
	1E.H8	IgG1 (K)	10^3	10^6	>4.5	>4.5
	6A.C3	IgG1 (K)	10^4	10^6	>4.5	>4.5
	1A.F10	IgG1 (K)	10^4	10^7	2.1	2.8
	1B.B5	IgG2b (K)	10^2	10^8	2.4	2.5
	1E.F9	IgG1 (K)	10^4	10^7	2.0	2.8
	6A.A6	IgG1 (K)	10^3	10^6	0.1	0.3
	1H.B1	IgM (K)	10^4	10^5	0.6	0.7
	1C.H2	IgG1 (K)	10^4	10^7	0.5	0.6
	1D.G3	IgG1 (K)	10^4	10^8	0.5	0.6
	1D.B12	IgG1 (K)	10^5	10^7	0.1	0.8
	1D.E6	IgG1 (K)	10^4	10^8	0.1	0.7
	1B.H11	IgG1 (K)	10^2	10^6	0.3	0.3
	1B.G4	IgM (K)	10^4	10^5	0.2	0.4
	1H.G5	ND[c]	10^1	10^3	0.1	0.2
	1C.H8	IgG2a, ND	10^5	10^6	0.0	0.9
	1E.C4	IgG1 (K)	10^3	10^5	0.0	0.0
N	3D.H10	IgG1 (K)	10^4	10^5	<0.3	<0.3
	3B.D10	IgG1 (K)	10^3	10^5	<0.3	<0.3
	3C.E4	IgG1 (K)	10^4	10^6	<0.3	<0.3
	3B.D8	IgG2b (K)	10^4	10^5	<0.3	<0.3
	3B.B5	IgG1 (K)	10^4	10^5	<0.3	<0.3
	3C.D8	IgG1 (K)	10^4	10^6	<0.3	<0.3
	3D.C10	IgG1 (K)	10^4	10^6	<0.3	<0.3
	3B.H3	IgG1 (K)	10^4	10^6	<0.3	<0.3
	3B.B6	IgG1 (K)	10^3	10^8	<0.3	<0.3
	3B.B4	IgG1, ND	10^4	10^8	<0.3	<0.3
E1	3B.D3	IgG1 (K)	10^4	10^7	<0.3	<0.3
	3B.B3	IgG1, ND	10^5	10^8	<0.3	<0.3
	3D.E3	IgG2b (K)	10^5	10^7	<0.3	<0.3
UK[d]	1A.D9	IgG2a, ND	10^2	10^4	1.0	0.8
	1G.E5	ND, K	10^1	10^4	0.0	0.1
	1G.H2	IgG2a (K)	10^3	10^4	0.0	0.9
	1E.D2	ND	10^4	10^6	0.0	0.0
	2C.D10	IgG1, ND	10^5	10^8	0.2	0.4
	2B.C2	IgG1, ND	10^5	10^8	0.1	0.1
	3D.B5	IgG1, (K)	10^4	10^8	<0.3	<0.3
	5B.H1	IgG2a, (K)	10^4	10^6	0.2	0.1
	6B.B10	IgG1, ND	10^4	10^6	0.0	0.2

[a] Titers were determined by RIA as described in Materials and Methods.
[b] The neutralization index was determined by dividing the number of PFU of virus per milliliter mixed with normal medium by the number of PFU of virus per milliliter in the presence of a MAb and is expressed as the $\log_{10}$ of this ratio.
[c] ND, Not determined.
[d] UK, Unknown.

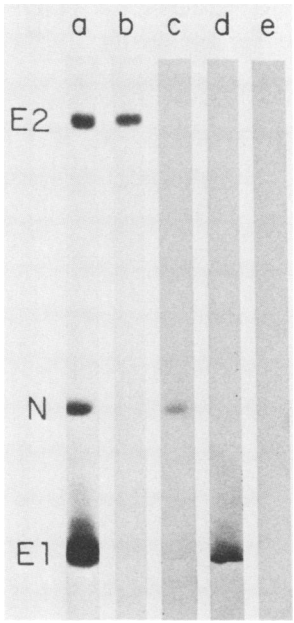

FIG. 3. PAGE of proteins from TGE virus particles recognized by representative MAbs. Purified [^{35}S]methionine-labeled TGE virus particles were dissociated with Nonidet P-40–sodium deoxycholate–disodium EDTA and, after selection in the immunoadsorption assay with MAbs specific for protein E2 (1D.E7, lane b), protein N (3B.D8, lane c), and protein E1 (3B.B3, lane d) or culture medium from P3-X63/Ag8 myeloma cells (lane e) (30), were subjected to SDS-PAGE in an 8% gel. Lane a, Proteins from TGE virus. After electrophoresis the gel was processed for autoradiography by the method of Chamberlain (10).

TABLE 4. Induction of neutralizing antibodies by native and denatured TGE virus[a]

Antigen	Treatment	Titer	
		RIA	Neutralization
TGE virus		$10^{3.5}$	$>10^{3.5}$
E2 protein	NP-40 + NaDOC[b]	10^2–10^3	$>10^{3.5}$
TGE virus	SDS[c]	$10^{3.5}$–$10^{4.2}$	$<10^{0.3}$
TGE virus	SDS + β-ME[c]	$10^{3.5}$–$10^{4.5}$	$<10^{0.3}$
E2 protein	SDS + β-ME[d]	10^2–10^3	$<10^{0.4}$

[a] Polyvalent antisera induced in groups of five mice immunized twice with 15 to 30 μg of the indicated antigen per dose.
[b] NP-40, Nonidet P-40; NaDOC, sodium deoxycholate.
[c] TGE virus incubated in 1% SDS–0.25% NP-40–0.5 M NaCl, in the presence or absence of 2.5% β-ME.
[d] E2 protein resolved by SDS-PAGE administrated with the gel.

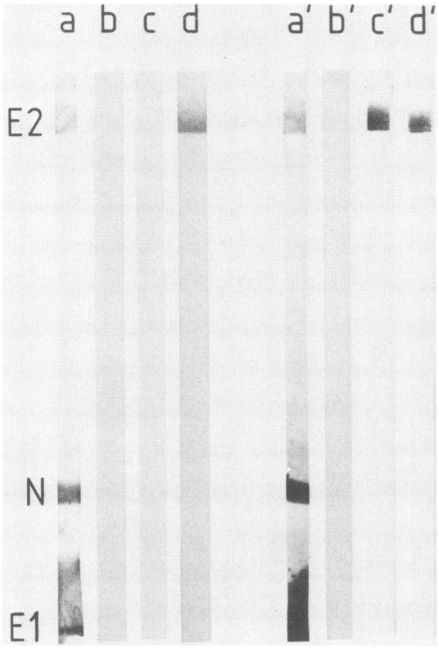

FIG. 4. Immunoblotting of TGE virus-specific mouse antiserum or MAbs with the viral proteins electrophoresed under reducing and nonreducing conditions. Proteins resolved by electrophoresis in the presence of SDS and β-ME (a,b,c, and d) or SDS alone (a', b', c', and d') and transferred to nitrocellulose blots were incubated with TGE virus-specific mouse antiserum (a, a') or serum from nonimmune mouse (b,b'), neutralizing (1G.A7) (c,c'), or nonneutralizing (1D.G3) MAb (d,d'). Antigen-antibody complexes were detected with peroxidase-conjugated mouse immunoglobulin-specific antiserum and 4-chloro-naphtol (25).

these epitopes must be conserved on different natural isolates. To study critical epitope conservation, the neutralization of 11 different TGE viruses by 14 NMAbs was studied. The results (Fig. 1) indicate that all six different critical epitopes studied (see below) were conserved in the clones of American (PUR 54, HOR 49, NEB 82, VAC-MVP, MIL 65), European [BRE 79 (D52)], or Japanese (SHI 56-19, SHI 56-25, SHI 56-83) origin with the exception of the Japanese isolate TOY 56, in which the epitopes defined by MAbs 1H.C2 and 1E.F9 were completely or partially modified, respectively. The presence of all the epitopes tested was confirmed in the PUR 54-C1.P30 virus (Fig. 1), which was derived from clone PUR 54-C1.P1 after 30 passages in ST cells.

TABLE 5. Reactivity of E2 glycoprotein-specific MAb with denatured TGE virus proteins

MAb or serum	Neutralization index[a]	Reactivity[b] after treatment	
		SDS	SDS + β-ME
1D.G3	<0.4	+	+
6A.A6	<0.4	+	−
1D.B12, 1B.H11, 1D.E6	<0.4	−	−
1G.A7	>4.5	+	−
1A.F10	2.0	+	−
1B.B5	2.0	−	−
1D.B3, 1D.E7, 1G.A6, 1H.C2, 1H.D2, 1C.C12, 1B.B11, 1E.H8, 1B.C11, 1B.H6, 6A.C3, 1E.F9	>4.5	−	−
Mouse anti-TGE virus serum	>4.5	+	+
Mouse nonimmune serum	<0.4	−	−

[a] The neutralization index was determined by dividing the number of PFU of virus per ml mixed with normal medium by the number of PFU of virus per ml in the presence of MAb and is expressed as the $\log_{10}$ of this ratio.
[b] Determined in the immunoblotting assay.

Number of critical epitopes in TGE virus neutralization. The 6 epitopes that could be differentiated by their sensitivity to denaturation with detergents and β-ME, their presence in the TOY 56 virus isolate, and the frequency of the corresponding *mar* mutants are summarized in Table 6. The main differential characteristic of each viral clone has been indicated with an asterisk.

DISCUSSION

To study the critical epitopes involved in TGE virus neutralization, we have developed a procedure to purify the virus to an extent higher than 95%, as determined by SDS-PAGE, isopycnic centrifugation, negative staining for electron microscopy, and prelabeling of infected cells with [^{35}S]methionine (Correa et al., submitted for publication). We have shown that TGE virus is composed of three proteins of relative molecular weights 200,000, 48,000, and 28,000 (Fig. 2), in agreement with other authors (28, 32). The 30-kilodalton (kDa) protein presented heterogeneity (from 28 kDa to 32 kDa) on the SDS-PAGE analysis, as described previously (19, 28, 32), which was due to different degrees of glycosylation, rather than differences in the amino acid sequence, as endoglycosidase H treatment yielded a single band of 28 kDa (28). In addition, the heterogeneous glycoprotein must have the same apoprotein since the E1-specific MAbs were able to bind in the immunoadsorption assay to polypeptides with relative molecular weights ranging from 28,000 to 32,000 (Fig. 3; results not shown).

E2 glycoprotein was purified from detergent-dissociated TGE virus by affinity chromatography with the MAb 1D.G3. This procedure was based on the resistance of the epitope recognized by this MAb to SDS (Fig. 4).

A panel of 48 TGE-specific MAbs has been described (Table 3). The viral proteins recognized by 38 of these MAbs were identified by the immunoadsorption assay. In addition, MAb 1B.C11 is probably E2 specific, as it neutralized TGE virus, although it did not recognize E2 protein either in the immunoadsorption assay or in the immunoblotting assay.

The numbers of MAbs that were specific for E2, N, and E1 proteins of TGE virus were 26, 10, and 3, respectively, suggesting that E2 protein was antigenically dominant. A

TABLE 6. Critical epitopes in TGE virus neutralization

MAb	Sensitivity[a] to:			Epitope presence[b] in TOY 56	Frequency of *mar* mutant[c]
	NP-40	SDS	SDS + β-ME		
1B.C11	+*	+	+	+	10^{-7}
1G.A6	−	+	+	+	10^{-6}*
1B.B11[d]	−	+	+	+	10^{-9}*
1G.A7	−	−*	+	+	10^{-6}
1H.C2	−	+	+	−*	ND
1E.F9	−	+	+	±*	10^{-6}

[a] Determined by studying the reactivity of the MAb in the immunoblotting and immunoadsorption assays. NP-40, Nonidet P-40; +, sensitive; −, not sensitive; *, relevant differential characteristic.
[b] Determined by the ability of the MAb to neutralize the TOY 56 clone of TGE virus. +, Presence; −, absence; ±, partially modified; *, relevant differential characteristic.
[c] Determined by *mar* mutant isolation. ND, Not determined; *, relevant differential characteristic.
[d] The putative *mar* mutant defined by this MAb has not been isolated due to its low frequency.

similar conclusion was obtained by Laude et al. (32) who, during the preparation of this publication, reported the obtainment of a collection of TGE virus-specific MAbs. Although the MAbs described in this report were not selected for their capability to neutralize the virus, 61 and 33% of the MAbs reacted with E2 protein or with all the viral proteins, respectively, suggesting that the critical epitopes were highly immunogenic. The TGE virus NMAbs described by Laude et al. neutralized viral infectivity up to 10^4-fold (32). Several MAbs described in this publication neutralized from 10^6- to 10^9-fold (all viral infectivity added to the neutralization assay) and possibly recognized an epitope distinct from the ones described previously, since the neutralizing capability of a MAb normally resides in its specificity (14, 15, 37).

Based on the different characteristics of the NMAbs, we have defined six critical epitopes (Table 6) of which only the one defined by MAb 1G.A7 was resistant to SDS denaturation, but only in the absence of β-ME. The fact that no neutralizing antiserum was induced in BALB/c mice after virus denaturation with SDS suggested that all critical (Table 4) epitopes in neutralization were conformation dependent, in agreement with several authors (Sachs et al. [49], Berzofsky et al. [3, 4], and Benjamin et al. [2]) who established that even sequential sites bind with highest affinity in a preferred conformation and, in this sense, that all antigenic determinants must be conformational (2–4).

Of the three critical epitopes defined in mouse hepatitis virus 4 (strain JHM), another coronavirus, two are sensitive to SDS denaturation, but one is at least partially resistant (9, 55). A similar situation exists in other viruses such as picornavirus (42), herpes simplex virus (12), and tick-borne encephalitis virus (26). On the latter virus, two critical epitopes have been identified, one of which is apparently resistant to SDS denaturation, but the reactivity of this epitope was lost when treatment was performed with SDS, β-ME, and carboxymethylation to prevent the reformation of disulfide bridges (F. Heinz, personal communication).

The frequency of TGE virus *mar* mutants selected with four MAbs ranged from 10^{-6} to 10^{-7}, lower than the frequencies described for other RNA viruses such as influenza virus (21), vesicular stomatitis virus (47), rabies virus (60), rotavirus (34), and poliovirus (40), for which the frequencies of *mar* mutants generally ranged from 10^{-4} to 10^{-6}. It was not possible to isolate *mar* mutants of the epitope defined by MAb 1B.B11, as this MAb always neutralized all infectious virus used in the assay. A similar situation has been described for vesicular stomatitis virus (36). MAb 1B.B11 could define an epitope essential for virus replication and may be related to a cellular recognition determinant common to several proteins, including Sindbis virus coat protein (46) and VP1 protein of several serotypes of foot-and-mouth disease virus (22).

The low variability predicted for the critical epitopes on TGE virus, based on the frequency of *mar* mutant isolation, was confirmed by studying the presence of those epitopes in viral isolates of American, Japanese, and European origin that were collected during an interval of at least 16 years. A similar conclusion was recently made by Laude et al. (32). In addition, 30 passages of a clone of PUR 54 in ST cells yielded a virus with the same critical epitopes of the original virus. These results are at variance with the ones described for other viral systems with a DNA genome, such as African swine fever virus, for which it was found that 20 passages were enough to detect epitope changes with 33% of the MAbs used (17).

The number of distinct critical epitopes involved in the neutralization of TGE virus (Table 6) was higher than those numbers reported for mammary tumor virus (37), rubella virus (59), infectious bronchitis virus (41), rabies virus (13, 60), strain JHM of mouse hepatitis virus 4 (55), and rhinovirus (48), which ranged from 1 to 4 epitopes, but was lower than those numbers found for herpes virus (57) and rhabdovirus (58) (7 and 9 epitopes, respectively). The number of critical epitopes in TGE virus was higher than the numbers described for other coronaviruses (mouse hepatitis virus and infectious bronchitis virus), although this may be due to the larger number of E2-specific MAbs that were used in the characterization of the critical epitopes and to the different criteria (sensitivity to detergents, frequency of *mar* mutants found for those epitopes, and their presence on different viral isolates) that we used in the present work.

Based on competitive RIA, the six critical epitopes could be assigned to two different antigenic sites: A, which included epitopes defined by MAbs 1E.F9, 1G.A7, 1H.C2, 1G.A6, and 1B.B11; and D, which included epitope 1B.C11 (I. Correa and L. Enjuanes, unpublished results). In addition, by reciprocal neutralization assays of the four *mar* mutants that we have been able to isolate with the six MAbs (Table 6), the epitopes were classified in three differentiable groups comprising MAbs (i) 1E.F9, 1G.A6, and 1G.A7; (ii) 1B.C11; and (iii) 1B.B11 and 1H.C2 (G. Jiménez and L. Enjuanes, submitted for publication). These results, based on two standard procedures usually used to define antigenic epitopes, allowed us to differentiate the epitopes recognized by each of the MAbs 1B.C11 and 1H.C2 from each other and from the ones recognized by MAbs 1E.F9, 1G.A6, and 1G.A7. In addition, the different sensitivities to SDS of the epitopes defined by the last three MAbs and their presence or absence in the TOY 56 isolate of TGE virus described in this publication allowed us to further differentiate the epitopes defined by these MAbs and to distinguish, in total, six different critical epitopes.

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